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Exhaled nitric oxide in patients with Wegener's granulomatosis

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ABSTRACT: In Wegener's granulomatosis (WG), a pathogenic role of infections, in particular of a chronic colonization of the nasal mucosa with *Staphylococcus aureus*, has been postulated. Nitric oxide (NO), which is thought to play a role in primary host defence and inflammation, is produced endogenously within the respiratory tract, mainly from the paranasal sinuses. In order to further characterize its role in WG, nasal and pulmonary NO excretion in WG patients in comparison to healthy volunteers was measured.

Seventeen patients with WG were included in the study. Five patients had active disease (bloody rhinitis with ulceration and crusting) and immunosuppressive therapy (IST), and 12 were in remission (six with, and six without, IST). *S. aureus* was found in the swabs of all patients with active WG and in three patients in remission. NO was measured in exhaled gas using a chemiluminescence analyser.

The NO excretion rate in nasally sampled gas was significantly reduced ($p<0.05$) in patients with active WG ((mean±SD)102±100 $nL \cdot min^{-1}$) compared to healthy controls (299±13 $nL \cdot min^{-1}$), and patients in remission (281±86 $nL \cdot min^{-1}$ with IST, 280±133 $nL \cdot min^{-1}$ without IST). Pulmonary NO excretion in active or nonactive WG patients did not significantly differ from that of healthy volunteers (48±21 $nL \cdot min^{-1}$).

These results demonstrate a reduced nasal NO excretion in active Wegener's granulomatosis. This may be caused by destruction and/or functional impairment of sinus epithelium. The reduced NO concentration may well compromise host defence in the upper airways, thus contributing to colonization with *Staphylococcus aureus* and further promoting Wegener's granulomatosis.

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Wegener's granulomatosis (WG) is a necrotizing granulomatous vasculitis involving the upper and lower respiratory tracts and the kidneys. In the upper respiratory tract, the disease shows an ulcerating chronic inflammation with granulomas leading to soft tissue necrosis and cartilage and bone destruction when left untreated. In patients treated with immunosuppressive agents comprising cyclophosphamide and high dose steroids, remission or marked improvement is achieved in ~90% [1]. However, relapses occur in half of the patients [1]. Antineutrophil cytoplasmic autoantibodies (ANCA) were found in most of the patients [2, 3], and clinical and *in vitro* observations have suggested a pathogenetic role of these antibodies [4]. A promoting influence of infections has been postulated, as exacerbation of WG is frequently known to follow viral or bacterial respiratory tract infections, and most patients give a history of flu-like symptoms shortly before the onset of vasculitic manifestation [5, 6]. Moreover, some authors describe an increased incidence of the disease in the winter months [6, 7]. It is thought that the infection leads to a priming of leukocytes and monocytes which are then activated by ANCA binding [4], leading to the release of oxygen radicals and proteases able to injure endothelial cells [4]

The gas nitric oxide (NO) is produced in mammalian cells by specific enzymes and is believed to play a vital role in many biological events, including regulation of blood flow, platelet function, immunity and neurotransmission [8]. It has been shown that NO is present in the exhaled air of humans [9] and that it is produced mainly in the upper airways [10, 11]. A continuous high production of NO takes place in the sinuses [12], and this NO enters the nasal cavity through the sinus ostia. Nasally released NO is inhaled with the respiratory gas and is absorbed to a considerable extent within the lower respiratory tract [11]. Regarding the physiological effect of NO in the airways, it has been suggested that NO is involved in primary host defence and gas exchange [11, 13]. The concentration of NO in normal paranasal sinuses and even in the nasal cavity, exceeds NO concentrations that are bacteriostatic, *e.g.* to *Staphylococcus aureus* [14]. Moreover, NO produced by the inducible nitric oxide synthase (iNOS) has been implicated in the pathogenesis of inflammation, [15]. It has been shown that NO synthesis is clearly enhanced at sites of inflammation, and inhibition of NO synthesis, *e.g.* by L-arginine analogues, may attenuate the tissue damage caused by the inflammation, in certain experimental models [16]. ALVING *et al.* [10] reported increased NO levels in the orally exhaled air of asthmatics, a finding that has been confirmed by others [17]. Elevated levels have also been reported in patients with lower and upper airway infections [10, 11, 18].

In patients with WG, a vasculitic inflammatory process in the upper airways could lead to increased NO synthesis,

which may play a role in the tissue destruction occurring in these patients. To study this hypothesis, NO excretion was measured in patients with active and inactive WG (with and without immunosuppressive therapy (IST)) and compared to the values of normal healthy volunteers.

Patients and methods

Patients

Seventeen patients (14 males and three females aged 29–74 yrs, mean 53 yrs) with WG were studied. The diagnosis was made according to the definition of the international consensus conference at Chapel Hill [19] and all patients fulfilled the American College of Rheumatology (ACR) criteria for WG [20]. Diagnostic criteria for WG were a typical clinical presentation with an involvement of the upper respiratory tract with bloody rhinitis with ulceration and crusting together with positive classic (c)-ANCA with cytoplasmic staining and/or granulomatous inflammation in the histology. Organ involvement at diagnosis was: kidney (88% of patients); ear, nose, throat (ENT) (100%); lung (76%); joints (65%); skin (35%); eye (35%); nervous system (29%); and others (12%). All but three patients had granulomas in a biopsy of the respiratory tract at diagnosis or later. The mean±SD disease extent index according to REINHOLD-KELLER *et al.* [21] was 9.8±2.5. The first disease manifestation occurred 7–227 months (mean 70±58 months) before the study and all patients had received immunosuppressive agents, including steroids (n=17) and cyclophosphamide (n=16), during previous treatment.

At the time of the study, five patients showed active disease (group A) with vasculitis manifestation in the upper airways (n=5), lung (n=3), kidney (n=1), joints (n=1), skin (n=1) and general symptoms (n=3) and received prednisolone (0.25–1 $mg \cdot kg^{-1} \cdot day^{-1}$) and cyclophosphamide (n=4) or methotrexate (n=1). The other 12 patients were in remission. Six of them still received IST (group B) (steroids (n=5), cyclophosphamide (n=1), cyclosporine (n=3)) with three of them being treated with trimethoprim/sulfamethoxazole. Six patients were not receiving IST (group C). All patients were nonsmokers except two who smoked 1–4 $cigarettes \cdot day^{-1}$. Apart from three patients in group B who received trimethoprim/sulfamethoxazole therapy and one further patient who received roxithromycin, no patient was treated with antibiotic therapy for at least 8 weeks before the study.

Five healthy volunteers (5 males, 23–27 yrs, mean 25±2 yrs) were studied as controls. The study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association. Informed consent was obtained from each patient and all healthy volunteers after institutional ethical approval.

Clinical examination

An endoscopic otolaryngological examination was performed in all patients. A nasal swab was taken from the inner cavity of the nose.

High-resolution computed tomography (HRCT) was performed if otolaryngological examination revealed any abnormalities that might point to disease activity or sinus infection. Examination of the chest was carried out in every patient and chest radiographs were performed at the time of the study in all patients with active WG.

Previous surgical interventions

An endoscopic pansinus operation had been performed in two patients (both group B), including a mastoidectomy in one. In a third patient with active disease at the time of the investigation, one total endoscopic pansinus operation and several small ones, involving only the maxillary sinus, had been performed. All these interventions had been carried out at least 12 months prior to the study.

NO-measurement

Patients and healthy volunteers inhaled air, from which NO had been eliminated by a zero air generator (PAG003; ECO Physics, Duernten, Switzerland) (NO <0.2 parts per billion (ppb)). Air was introduced in excess into a tube *via* a reservoir consisting of a 5 L rubber bag. A gas source with minimal positive pressure was maintained by a second tube system connected in a side stream. Inspiratory and expiratory gases were divided by a two-way non-rebreathing valve (Intersurgical, St. Augustin, Germany), which was connected to either a mouthpiece or a tightly fitting nasal mask (Respironics Inc., Murrysville, PA, USA). Exhalation was performed into a 1 m long silicon tube containing a sampling port for NO analysis, and distally occluded by a flap valve to prevent retrograde contamination with ambient air. Respiratory flow rates were monitored using a pneumotachograph (PT36; Jaeger, Würzburg, Germany).

NO was measured using a highly precise chemiluminescence analyser (CLD 780 TR; ECO Physics) with a rise time <0.5 s for 500 ppb NO and a lower detection limit of 0.05 ppb for a 1-min integration interval. The unit ppb corresponds to a volume fraction of 10^{-9}. Due to a pre-reaction chamber, the analyser is able to correct the measured values for the cross sensitivity against hydrocarbons. Calibration was performed with a certified test gas containing 205 ppb NO dissolved in N_2 (AGA, Bottrop, Germany).

NO was measured in exhaled gas while breathing was exclusively nasal *via* a mask covering the nose (case A), or oral *via* a mouth piece (case B). In order to prevent admixture of nasal NO during oral breathing, the subjects were instructed to close their soft palate. In addition, nasal gas was continuously sampled with a flow rate of 1.4 $L \cdot min^{-1}$ *via* a thin catheter connected to an olive, which fitted tightly into one nostril (case C). Due to voluntary closure of the soft palate, the gas flow was directed from the open nostril through the nasal cavity into the catheter. Thus, nasally released NO was diluted with ambient air.

NO concentrations and respiratory flow rates were recorded online using a computerized data acquisition system and averaged over a time interval of 1 min. NO excretion rates were calculated according to the following formula, and expressed in nanolitres per minute:

$$\text{NO rate } (nL \cdot min^{-1}) = \Delta\text{NO concentration ppb} \times \text{flow rate } L \cdot min^{-1}.$$

where ΔNO concentration denotes concentration differences (expired gas-inspired air (cases A and B); nasally sampled gas-ambient air (case C). In cases A and B, the

flow rate corresponds to the minute ventilation, while in case C the sampling flow rate was inserted in the above relationship.

Statistical analysis

Data are expressed as the mean±SD. Differences in the NO concentration in the exhaled air were assessed by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using the Bonferroni test. Pearson's correlation coefficient was computed to test the association between variables. A p-value <0.05 was considered to indicate statistical significance.

Results

Clinical examination

One patient in group C showed a perforation of the nasal septum (known since 1991) with dry, slightly bloody skin at the septum. Apart from that, examination of the ENT was uneventful. HRCT of this patient was normal. A second patient (group C) showed mild mucosal changes in the nasal and paranasal cavity and an ulcer of the external ear canal which resolved 2 weeks later under local treatment. In all other patients of group B and C, the ENT examinations and lung examinations were uneventful. In the group with active WG, group A, all patients showed bloody rhinitis with ulceration and crusting. Two patients had known perforation of the nasal septum. Sinus involvement was found in four patients. Lung examination revealed faint crackles in one patient and was uneventful in all others.

High resolution computed tomography of the paranasal sinuses

Changes in the HRCT were seen in four patients with active disease (group A). Pathological findings included opacity in the paranasal sinuses (all four patients, but two to a moderate extent), (probably due to mucosal thickening of the paranasal sinuses and/or granuloma formation), the nasal cavity (four patients but one to a moderate extent), the ethmoidal cells (one patient) and the mastoid cell system (one patient). Bone erosion and/or destruction was found in three patients.

Nasal swabs

S. aureus were found in all patients with active WG, in two patients together with other bacteria (*Pseudomonas, Escherichia coli*). Three of six patients of group B had *S. aureus*, one patient had *Pneumococci.* In group C, no *S. aureus* was found in the swabs.

Chest radiograph

In two patients with active WG (group A), chest radiograghs showed multilocular nodules and/or cavitations, together with patchy opacifications in one patient. In a third patient, reticular markings and ill-defined nodular opacities were found.

Nitric oxide measurements

The results of NO measurements in exhaled gas are summarized in table 1. Orally exhaled NO concentrations in healthy controls (4.7±1.4 ppb corresponding to an excretion rate of 48±21 $nL \cdot min^{-1}$) were not significantly different from those of WG patients in groups A, B or C. The NO excretion rate during nasal breathing in healthy controls was significantly different from that of active WG patients (group A) ($p<0.05$), but not from that of WG patients in groups B and C (table 1). NO concentrations in nasally sampled gas are shown in figure 1. (In this case NO concentrations were higher compared to those in nasal breathing, due to missing absorption of inhaled nasal NO in the lower respiratory tract). The corresponding excretion rates are shown in figure 2. The values in active WG patients, group A (102±100 $nL \cdot min^{-1}$), were significantly lower ($p<0.05$) than in healthy controls (299±13 $nL \cdot min^{-1}$) and in WG patients in remission (281±86 $nL \cdot min^{-1}$ group B; 280±133 $nL \cdot min^{-1}$ group C).

The patient with the lowest NO concentration in group B had a pansinus operation with radical removal of the sinus epithelium. The patient with the lowest NO concentration in group C had a septum perforation with bloody skin. Clinically a sinus affection was suspected but the HRCT scan was normal. In this patient, clinical signs pointed to a smouldering active WG. As the patient was first classified in the group with inactive disease the authors did not want to change this classification afterwards.

In two WG patients with acute disease, NO concentration in nasally sampled gas did not exceed the NO concentration of ambient air, and exhaled NO concentrations were almost identical during nasal and oral breathing (3.4 *versus* 3.3 ppb; 3.9 *versus* 4.7 ppb, respectively). Both patients had a severe involvement of the upper airways, one patient with a long-lasting destructive vasculitic process and the second patient with severe acute necrotic lesions.

Table 1. – Nitric oxide concentration and excretion rate in healthy controls and Wegener's granulomatosis (WG) patients during oral and nasal breathing

		Patients with WG		
	Healthy controls	Group A	Group B	Group C
Subjects n	5	5	6	6
NO concentration ppb				
Orally expired	4.7±1.4	3.9±1.7	5.2±2.4	4.5±2.1
Nasally expired	18.3±2.3	10.9±6.7	25.9±13.6	22.6±14.9
NO excretion rate $nL \cdot min^{-1}$				
Orally expired	48±21	29±20	41±15	28±13
Nasally expired	197±27	87±56*	185±51	168±91

Data presented as mean±SD. Group A: patients with active WG; Group B: patients in remission, receiving immunosuppressive therapy (IST); Group C: patients in remission not receiving IST; ppb: parts per billion. *: $p<0.05$ when compared to healthy control subjects.

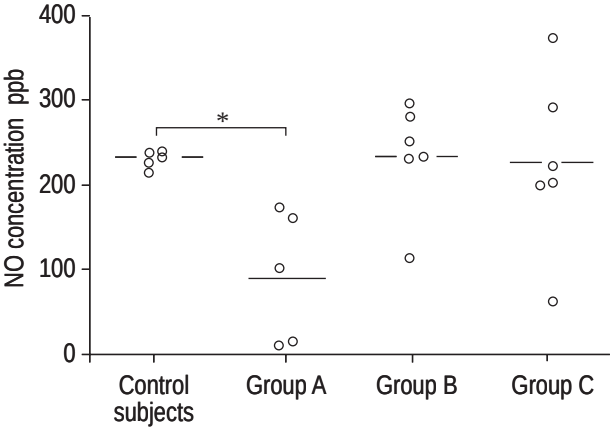

Fig. 1. – Nitric oxide concentrations in nasally sampled gas in Wegener's granulomatosis patients and healthy control subjects. Horizontal bars represent the mean values of each group. For definitions of groups, see footnote to table 1. ppb: parts per billion. *:p<0.05.

There was no correlation between NO excretion rates determined in orally expired gas and in nasally sampled gas (r=0.29, p=0.19) and no correlation of either of these with age (r=-0.20, p=0.37 and r=-0.18, p=0.41, respectively). A strong correlation was found between NO rates in nasally expired gas and NO rates in nasally sampled gas (r=0.88, p<0.001).

Discussion

In this study, exhaled nasal NO was not increased by the vasculitic inflammation, but was significantly reduced in patients with active WG. In patients in remission with or without IST, NO levels were normal compared to healthy volunteers. In the three patient groups, orally exhaled NO (measured during closure of the soft palate, representing NO concentration in the lower airways) was not different from that of normal control subjects. Successful separation between nasal and pulmonary fractions of NO is indicated by the missing correlation between NO rates measured in nasally sampled and orally expired NO gas.

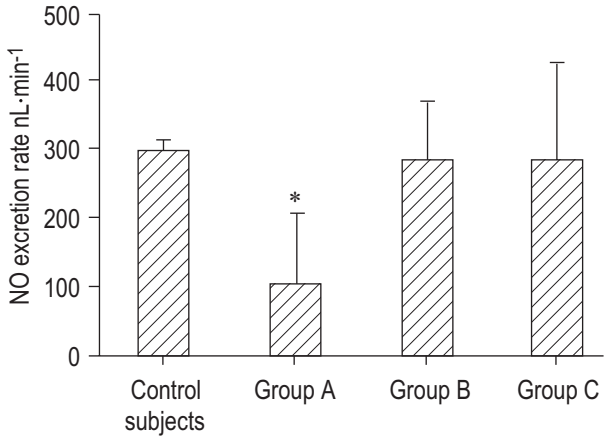

Fig. 2. – Nitric oxide excretion rate in nasally sampled gas in Wegener's granulomatosis (WG) patients and healthy control subjects. For definitions of groups, see footnote to table 1. *: p<0.05 compared with healthy control subjects and WG patients in remission (Groups B and C).

The NO concentrations measured in the control group are comparable to the findings of other studies. ALVING *et al.* [10] reported an exhaled NO concentration of 23±2 ppb (mean±SEM) in 12 healthy adults, when they breathed nasally through a face mask. LUNDBERG [16] measured 270±37 ppb NO in the nasally sampled gas of four healthy adults, using a flow rate of 0.8 $L \cdot min^{-1}$. In 10 intubated healthy adults, GERLACH *et al.* [11] reported 3–5 ppb in the expiratory limb of the ventilator.

The present results lead to the conclusion that inflammation in the upper respiratory tract in patients with WG does not lead to increased NO production of the sinus epithelial cells. On the contrary, patients with active WG seem to have a reduction in NO production, which is probably not a constitutional phenomenon in this patient group, as patients in remission have exhaled NO levels comparable to those of healthy control subjects. The results of orally exhaled NO do not provide evidence that WG manifestation in the lung leads to changes in exhaled NO levels. However, it has to be admitted that only three patients had lung involvement, two of them with nodules and cavities, which is a localized process. In addition, treatment with steroids may lead to reduced NO levels in the lower airways [22]. Therefore, the authors cannot exclude the possibility that severe vasculitic lung manifestation with widespread infiltrates may influence pulmonary NO excretion.

The reason for the reduced nasally exhaled NO values might be a destruction and/or functional impairment of the sinus epithelium as a result of the vasculitis and necrotic inflammation leading to a dysfunction in mucosal NO synthesis. In addition, mucosal swelling and thickening (shown by HRCT) may reduce the passage of NO from the nasal sinuses, the major place of NO production [16], to the nasal cavity. In children with Kartagener's syndrome (a triad consisting of situs inversus, sinusitis and bronchiectasis), who generally have mucus-filled paranasal sinuses, NO is almost absent, and nasal NO excretion is decreased by two-thirds in patients with cystic fibrosis (CF) [16, 23]. Whether the reduced or absent NO in exhaled air in these patient groups reflects reduced diffusion of NO from the paranasal sinuses into the airway lumen or a dysfunction in mucosal NO synthesis is not clear. The colonization with *S. aureus* itself does not seem to be responsible for the reduced nasal NO excretion in active WG patients. Thus, in long-term intubated patients (without sinusitis), GERLACH *et al.* [11] demonstrated the highest nasal NO concentrations among those patients with *S. aureus* infection. In the patients with active WG no clinical evidence of active bacterial sinusitis leading to reduced NO excretion [24] could be shown. In addition, those patients were treated with immunosuppressive drugs with the resultant clinical improvement. Under this treatment, bacterial sinusitis would have shown severe progression.

Reduced levels of NO will probably compromise host defence in the upper airways [13]. Children with Kartagener's syndrome or CF have severe problems with recurrent airway infections, and patients with CF frequently suffer from chronic colonization by *S. aureus* [25]. Therefore, the high incidence of *S. aureus* in the nasal swabs, described in patients with WG [26] and also found in patients with active disease from the present study, may reflect the higher susceptibility to infections as a result of

the reduced presence of NO; infections may trigger a further increase in disease activity. It has been shown that the *S. aureus* colonization in WG is associated with an increased risk of a relapse involving the upper respiratory tract [26]. IST does not seem to be involved in the reduced nasal exhaled NO values in active WG, as patients in remission receiving IST had normal NO levels. Moreover, it has been demonstrated that NO synthase in the sinus mucosa is not downregulated by systemic steroids [27].

The finding that the administration of L-arginine increased nasal NO levels [16, 27] may have therapeutic implications during active WG. It might be speculated that L-arginine supplementation may increase resistance to airborne infectious agents. This might also be important regarding the risk of opportunistic infections involving the lung, as NO has been shown to increase ciliary beat frequency in airway epithelium both *in vitro* [28] and *in vivo* [29].

However, serial exhaled nitric oxide determination in patients with active disease and following successful immunosuppressive therapy is necessary before therapeutic intervention should be planned. Moreover, these measurements might show if exhaled nitric oxide can be used for a noninvasive follow-up in patients with Wegener's granulomatosis to control therapeutic success and/or the onset of relapses.

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